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IN VITRO production of ANNATTO (Natural food dye) from *BIXA ORELLANA* L.

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ABSTRACT:
As color is often a key consumer perception for food preference and acceptability, alimentary colorants play a key factor in the food Industry. Due to the consumer awareness and concern for health in recent years, the number of dyes suitable for use in food has been drastically reduced and natural food colorants are gaining popularity. Annatto is one of the natural colorants used in confectionery. This paper aims to explore the possibility of obtaining the dye-annatto from callus of different parts - root, stem, leaf and seeds of the natural dye yielding plant *Bixa orellana*, *in vitro* techniques and for finding out the efficacy of different wavelengths of light on callus formation and carotenoid production.

Key words: *Bixa orellana* L., Callus, Color, Consumer awareness, Food industry, Natural Food Dye.

INTRODUCTION

Higher plants represent a valuable resource for a great variety of chemicals or secondary metabolites of pharmaceutical importance.

These higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragnance ingredients, food additives and pesticides [1]. The natural dye yielding plants are *careopsis tinctoria, Galium Verum, Genista tinctoria, Indigofera suffruticosa, Isatis tinctoria, Reseda luteola, Rubia tinctorium & Bixa orellana L. Bixa orellana* L., a tropical plant belonging to bixaceae is cultivated in different parts of the world for the food dye. The dye is reported to be present as a red-orange pulp in the outer covering of the seed [2]. Bixin and norbixin are the principle coloring constituents of annatto. This dye has been proved non-carcinogenic & is used in food, drugs & cosmetics. The dye is used in dairy industry for coloring butter, cheese, ghee, chocolate, ice cream and in textile industry for dyeing cotton, silk clothes, leather and in coloration of medicines & in making boot polishes and so also in the preparation of bindhi or kumkum. It is a multipurpose species whose bark, leaves, roots and seeds are used for medicinal, pharmaceutical and edible coloring purpose [3].

Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a Phyto chemical product by manipulation of the chemical or physical microenvironment to produce a compound of potentially more value for human use. The plant tissue and cell culture technology has proved beyond doubt the production of secondary compounds, which are useful as drugs, food additives, edible colors for food and medicines. These secondary products can now be successfully accumulated in many undifferentiated plant cell cultures (callus and suspensions). Hence, In the light of the current consumer demands for natural food products and plant-derived medications, *in vitro* production of valuable secondary products has become an industrially promising venture.

The present study aims to analyze the bixin content in the callus obtained from different plant parts- root, stem, leaf of *in vitro* grown seedlings and seeds (of parent plant) of *Bixa orellana* L., and the Qualitative analysis of the Dye-Bixin in the same.

MATERIALS & METHODS

Seeds of *Bixa orellana* L., were collected from creamish – pink capsule variety of N. R. Colony – N_1 accession and these seeds were germinated *in vitro* to obtain the seedlings. This accession was found to possess maximum amount of dye in its seeds.

The MS medium was chosen to assess the response of explants for callus induction and growth recommended by [4]. The media was steam sterilized in an autoclave at 121^0C and 108 k pa for 15 minutes.

PREPARATION OFPLANT MATERIAL

Seed/leaf/root/stem explants were washed in running water for 30 minutes. Then the explants were soaked in 15% Teepol for 15 minutes. These were then washed thoroughly in running water and transferred into 70% alcohol for 15 seconds, followed by dipping in previously sterilized 0.1% $HgCl_2$ for 15 minutes. This was

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IJBSAT (2012), 4(4):23-28

then washed for 4 times in double DW. Then the material was given a dip in H_2O_2 (Hydrogen peroxide) for 1 minute and then finally washed with sterile DW for 4 times to remove all traces of the sterilant.

CALLUS INDUCTION

Experiments were conducted to induce callus from seeds of parent plant as well as root, stem and leaves excised from 4 weeks- old *in vitro* raised plants, derived from the seeds of *Bixa orellana* L., N_1 (N.R. Colony) accession.

The stem and root explants, measuring 0.5-1 cm and the leaf explants measuring 0.5–1.5cm were inoculated on to the Basal MS media supplemented with NAA (26.85 µM) and BAP (4.44 sµ M), 2, 4-D (4.52 µ M)and BAP (8.88 µ M), NAA(5.37 µ M) and BAP(13.32 µ M) and 2, 4-D (4.52 µ M) and BAP (13.32 µ M), NAA (5.37 µ M and 26.85 µ M), 2,4-D (4.52 µ M) and BAP (4.44 µ M, 8.88 µ M and 13.32 µ M).

INFLUENCE OF LIGHT QUALITY ON SEED CALLUS

Seed callus sub cultured on to the Basal MS media supplemented with NAA (5.37 µM) and BAP (13.32µM), were tested for its growth under different wavelengths of light (Red, Blue, Green, Yellow) and white light served as a control.

ESTIMATION OF DYE IN CALLUS DERIVED FROM EXPLANTS

Callus derived from different explants (root, stem, leaves and seeds) were analysed for total bixin content [5].

QUALITATIVE ANALYSES OF BIXIN FROM CALLUS DERIVED FROM *BIXA ORELLANA* L. PLANT PARTS

Thin layer chromatography was adopted for separating, purifying and quantifying the carotenoid pigment in the callus of the explants. Stationary phase of Silica gel. G grade with 13% $CaSO_4$ was used as a binder and the solvent system consisting of Benzene, Methanol and Ammonium hydroxide (3 : 6.5 : 0.5), was used for characterizing the dye.

RESULTS & DISCUSSION

On the MS basal medium supplemented with NAA (26.85µ M) and BAP (4.44 µ M) & 2, 4-D (4.52 µ M & BAP (8.88 µM), after 30 days of inoculation, all the explants callused that was friable and scanty. Later sub culturing was done on MS + 2,4-D (4.52µm) & BAP (13.32µM) and MS + NAA (5.37 µM) and BAP (13.32µM). The callus growth increased after 7 days of sub culturing. Callus was compact and brownish in case of root, leaves and seeds, while it was white and creamish in case of stem callus (Plates - 1, 2, 3, 4 and Table- 1).

PLATE 1 : (*In vitro* raised seedlings of *Bixa orellana* L. from seeds of N_1 accession)

ISSN: 0975 - 4539
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PLATE 2 : (Callus obtained from leaf, root and seed explants of N_1 accession of *Bixa orellana* L).

PLATE 3 : (Callus obtained from stem, leaf and root explants of N_1 accession of *Bixa orellana* L.)

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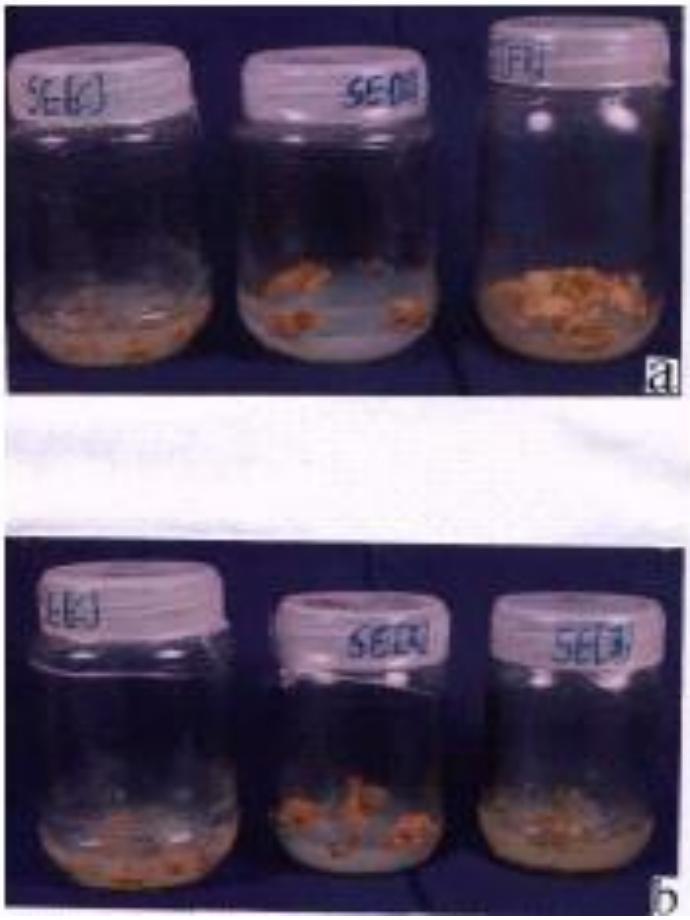

PLATE 4 :(Effect of red, far red and blue light on callus growth rate)

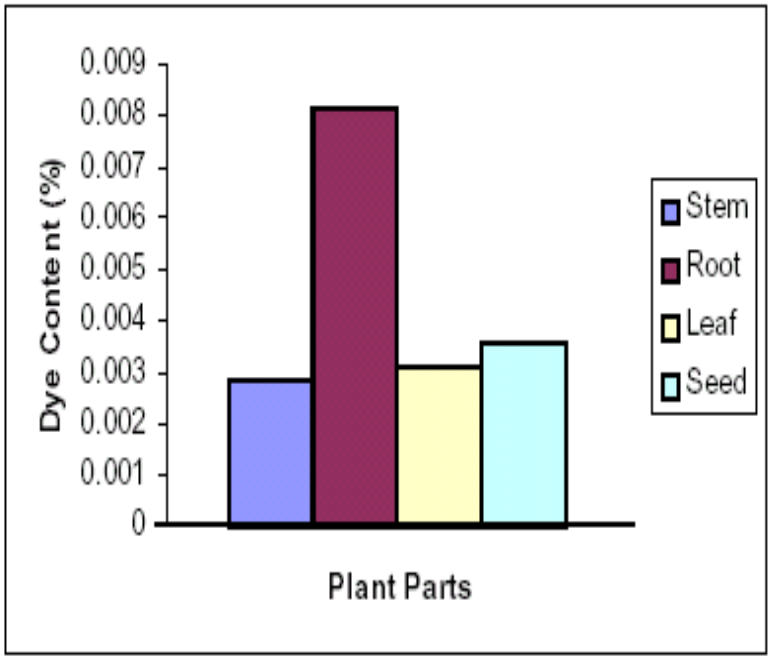

Fig 1 : (Dye content in callus obtained from different parts of *Bixa orellana* L. (N1))

Table-1: Effect of combination of Auxins and cytokinins for callus Induction from different explants of *Bixa orellana* L. (N_1 accession)

Sl. No.	MS + Growth Regulators (mg/L)	Explants			
		Root	Stem	Leaf	Seeds
1.	NAA (5mg/L) + BAP (1mg/L)	++	++	++	++
2.	2, 4-D (1mg/L) + BAP (2mg/L)	++	++	++	++
3.	NAA (1mg/L) + BAP (3mg/L)	++++	++++	+++ +	++++
4.	2, 4-D (1mg/L) + BAP (3mg/L)	++++	++++	+++ +	++++

++ = Moderate callusing
++++ = Profuse callusing

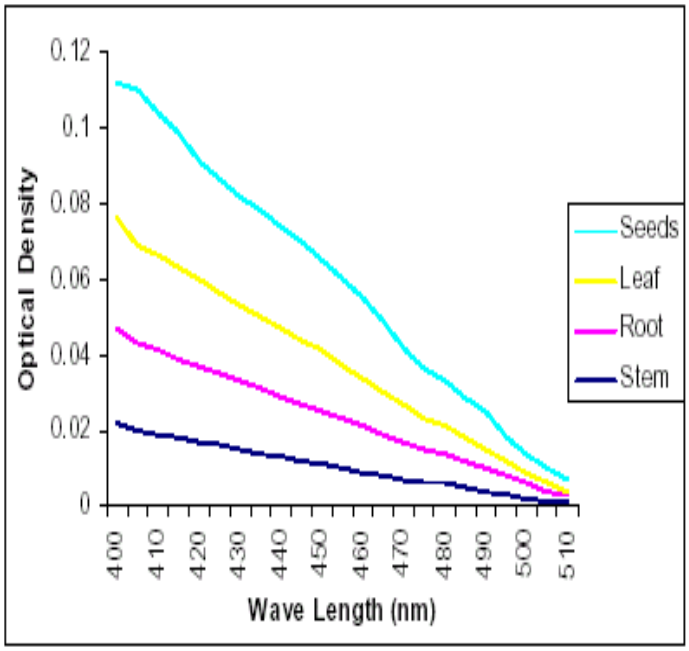

Fig 2: (Absorption Spectra of Carotenoid-1 obtained from Calli of stem, root, leaf & seeds of *Bixa orellana* L.(N_1 accession))

The quality of light, especially red and Far red increased the callus growth rate and the callus produced was very less under Blue light (Plate- 4). The callus obtained from all the explants (Seeds, roots, leaf and stem) showed the presence of bixin. Root callus had maximum amount of dye (0.00812%), followed by seed callus (0.00367%), leaf callus (0.0031%) and stem callus (0.0028%), as in (Fig -1).

The qualitative analysis of the dye obtained from the callus of different plant parts using TLC, showed good amount of carotenoid-1 (Table- 2)

The absorption spectrum of the purified carotenoid in methanol from callus of different plant parts was very similar but the amount of the pigment varied widely among all of them. They exhibited almost the same peak absorption too (Fig- 2).

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Hence, one doesn't have to wait till the fruiting time for collecting the dye from seeds. Callus production from plant parts can deliver the dye in a very short duration. By altering the hormonal composition and the light conditions one can achieve bixin of desired quality from the *Bixa orellana* L., cell cultures.

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